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A versatile promoter for the expression of proteins in glandular and non-glandular trichomes from a variety of plants

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Running Title: trichome expression promoter

Abstract

We have isolated a DNA regulatory fragment from the promoter region of the *OASA1* gene, encoding the cytosolic O-acetylserine(thiol)lyase enzyme that is highly expressed in *Arabidopsis thaliana* trichomes. This DNA fragment has been named as ATP fragment and comprises 1435 bp of the genomic region upstream of the *OASA1* gene and 375 bp of the transcriptional initiation start site, containing the first intron of the gene. The ATP fragment, fused to the green fluorescent protein (GFP) and β-glucuronidase (GUS) reporter genes, is able to drive high-level gene expression in *A. thaliana* trichomes. Deletion analysis of the ATP fragment determined that the region from –266 to –66 contains regulatory elements required for trichome expression. In addition, the region from +112 to +375, comprising the first intronic region of the gene, is also essential for trichome gene expression. Expression of the full-length ATP fragment in tobacco and peppermint shows that this fragment is also able to drive expression in glandular trichomes and suggests additional biotechnological applications for this promoter.

Key words: Arabidopsis, tobacco, peppermint, confocal microscopy

Introduction

Trichomes are specialized unicellular or multicellular structures derived from the epidermal cell layer. Unicellular non-glandular trichomes, such as those present in *A. thaliana*, are not able to produce or secrete phytochemicals but may function as defensive physical structures against herbivores (Eisner *et al*, 1998), as sinks for toxic heavy metals and xenobiotics (Domínguez.Solís *et al.*, 2004; Gutiérrez-Alcalá *et al.*, 2000) and regulating water absorption (Werker, 2000). Biotechnological applications of non-glandular trichomes have not been widely explored except in the case of cotton ovule trichomes that have agricultural importance in the production of fibers with enormous commercial value (Kim and Triplett, 2001).

Multicellular trichomes can be found in many different species and often form glands that secrete phytochemical compounds (e.g., organic acids, polysaccharides, terpenes, or salt) as well as secondary compounds such as those produced in trichome exudates (e.g., terpenoids, flavonoids, and phenylpropanoids) (Duke *et al.*, 2000). Glandular trichomes show variously forms and can be unicellular or pluricellular and morphological distinction can be observed between the apical and the basal part of the glands (Werker, 2000). Glandular secretory trichomes have potential biotechnological applications as a result of the great variety of phytochemical molecules produced. Many of these molecules have significant commercial application in the production of flavors and fragrances, such as vanillin and benzaldehyde (Krings and Berger, 1998), the pharmaceutical industry, such as artemisinin (Li *et al.*, 2002; Mahlberg and Kim, 1992), and in host defense or plant-

plant allelopathy (Werker, 2000). Extensive references about secreted molecules from plant trichomes are available (Callow, 2000; Wagner *et al.*, 2004). Approaches for exploitation of trichomes, for both commercial and agronomic purposes, require the application of appropriate molecular tools to attempt to modify the metabolic pathways in trichome cells. One of these tools is the development of trichome-specific promoters to direct gene expression in these cells. Although, strong and constitutive promoters, such as the 35S CaMV promoter are also highly expressed in trichomes, it could be problematic for expressing a potentially cytotoxic protein. The main advantage of a trichome-specific versus a constitutive promoter is the capability to bioengineer biochemical pathways present only in trichome cells without altering biochemical pathways in other tissues and to avoid affecting plant growth or productivity. This effect has been recently tested by the expression of defense-related gene in wheat epidermis under control of the epidermal-specific *GstA1* promoter from wheat. In this work, it is observed that tissue-specific expression of a potentially harmful transgene is superior to ubiquitous expression throughout the plant body (Alpeter et al, 2005).

The obtaining of trichome-specific promoter has been attempted after isolation of the *LTP3* gene promoter from cotton and the *CYP71D16* gene promoter from tobacco. These promoters were able to direct GUS expression, the former in non-glandular and the latter in glandular trichomes (Liu *et al.*, 2000; Wang *et al.*, 2002). The *CYP71D16* promoter was also successfully used to suppress cembratrieneols in trichome exudates and to reduce aphid infection in tobacco (Wang

et al., 2004a). However, a universal trichome promoter, able to direct high-level expression in a wide range of trichomes and plant species, has not been reported.

In this work, we report the isolation of a DNA regulatory fragment, able to direct preferentially high-level expression of GFP and GUS reporter genes, in non-glandular trichomes from *A. thaliana*, and in glandular trichomes from tobacco and peppermint. This trichome regulatory element was isolated from the *OASA1* gene from *A. thaliana*, encoding the cytosolic O-acetylserine(thiol)lyase enzyme involved in cysteine biosynthesis (Barroso *et al.*, 1995). This gene has been demonstrated, by *in situ* hybridization, to have high-level gene expression in *A. thaliana* trichomes (Gotor *et al.*, 1997; Gutiérrez-Alcalá *et al.*, 2000).

Materials and methods

Plant material and growth conditions

Wild-type *Arabidopsis thaliana* was grown on moist vermiculite supplemented with Hoagland medium or in soil media at 20°C in the light and 18°C in the dark, under a 16-h white light/8-h dark photoperiod, as previously described (Dominguez-Solis *et al.*, 2001).

Transformed or wild-type tobacco (*Nicotiana tabacum* cv. W38) seeds were grown in soil media at 26°C with a 16-h photoperiod. Peppermint (*Menta x piperita* L.) cultures were started from rhizome explants and maintained in growth chambers at 25°C with a 16/8-h photoperiod.

DNA cloning and plasmid construction

The ATP regulatory sequence was isolated by screening an *A. thaliana* genomic DNA library (~ 500.000 pfu) constructed in the EMBL3 vector using [α-^{32}P]dCTP-labelled *OASA1* cDNA (Barroso *et al.*, 1995). After analysis of several positive clones, a 2692-nucleotide DNA fragment, containing the promoter and 5´end of the gene, was isolated and cloned into the pBluescript KSII vector at the *Kpn I* restriction site. This clone was used as a template for PCR to obtain a shortened DNA fragment using the following primers: forward primer (5´-ATGCCCGGGTACCTACTGCAGTCCGGT-3) containing a *Sma I* restriction site (underlined); reverse primer (5´-ATGGGATCCCGAGGCCAT GATTCAAGC-3) containing a *BamH I* restriction site (underlined). The PCR-amplified fragment was digested with *Sma I* and *BamH I* and gel-purified. This fragment was ligated into the pBI121 vector (Clontech, USA), previously digested with *Hind III*, treated with Klenow enzyme, and digested again with *BamH I* to release the 35S promoter. The inserted DNA regulatory sequence consists of 1810 nucleotides and corresponds to the sequence between nucleotides 8521846 and 8520037 of *A. thaliana* chromosome 4. The resulting vector was named pATP-GUS. The GUS sequence was removed from the pATP-GUS vector by digestion with *BamH I* and *Sst I* prior to replacement with the smGFP gene (Davis and Viestra, 1998) at the same restriction sites to yield pATP-GFP.

Primer extension analysis

Total *A. thaliana* RNA was extracted from root tissue by using the RNeasy Plant Mini Kit (Qiagen GmbH, Germany). The 5´-end of the RNA was mapped by combining 2.5 pmol PE primer (5´-GAAACCGGCAGAG GAATAAGCAAGTG-3) and 10 µg RNA in a 10-µl final volume containing 1 mM Tris-HCl (pH 8), 1 mM EDTA, and 10 mM KCl. The mixture was incubated at 100°C for 10 min, cooled to 47°C, and further incubated for 1 h. The PE primer was extended using 500 U SuperScript II reverse transcriptase in the following reaction mix: 10 mM DTT, 25 µCi [α-^{32}P] dCTP (3000 Ci mmol^{-1}), 0.18 mM of dNTP mix (dATP, dGTP, dTTP) and SuperScript II enzyme buffer. After an incubation of 1 h at 47°C, 2 U of DNase-free RNase was added and the reaction incubated 15 min at 37°C. The reaction product was separated on a denaturing sequencing gel (6% polyacrylamide) in parallel with the sequencing reaction products of the ATP fragment using the PE primer.

Transformation of Arabidopsis, tobacco, and peppermint plants

For plant transformation, the chimeric gene constructs were transformed into *Agrobacterium tumefaciens* strain C58pMP90 (Koncz and Schell, 1986). *A. thaliana* (ecotype Columbia) was transformed by dipping the developing floral tissues into a solution containing the *A. tumefaciens* strain, 5% sucrose, and 0.005% (v/v) surfactant Silwet L-77, as previously described (Clough and Bent, 1998). Transgenic plants were recovered by selecting seeds on solid MS medium containing 50 mg l^{-1}

kanamycin. Copy number was assessed by monitoring the segregation of resistance to kanamycin. T_3, or subsequent generations of each line, were used for the experiments described in this paper.

M. x piperita L. explants were also transformed with *A. tumefaciens* strain C58pMP90 containing the pATP-GUS or GFP constructs and generated according to the methods described by Diemer *et al.* (1998). Tobacco (*N. tabacum* cv. W38) leaf disks were used for transformation using standard procedures (Gotor *et al.*, 1993).

Analysis of the GFP and GUS reporter genes

The accumulation of the GFP reporter protein was analyzed *in vivo* by laser confocal microscopy**.** Leaves were carefully cut into small pieces (4-9 mm^2) and mounted onto a slide using a spacer between the slide and coverslip to avoid crushing the trichomes. Samples were observed using Leica HCX PLAN-APO 63x 1.4 NA or HCX PLAN-APO 40x 1.25 NA oil immersion objectives attached to a Leica TCS SP2 spectral confocal microscope (Leica Microsystems, Germany). GFP was imaged using the 488-nm line of an argon ion laser, either in single confocal optical sections or serial optical sections of leaves. Emitted light was collected through a triple dichroic beam-splitter (TD 488/543/633) and simultaneously detected after spectral separation in the 493-540-nm range on the PMT1 for GFP imaging (pseudocolored green) and in the 541-600-nm range on the PMT2 for chloroplast and cell wall auto-fluorescence (pseudocolored red). Manual unmixing eliminated the strong chloroplast signal, which leaked from the red channel into the green. Image montages were assembled using Photoshop™ (Adobe Systems, Mountain View, CA, USA).

The histochemical assay for GUS enzyme activity was carried out according to the procedure of Jefferson *et al.* (1987). Leaves were carefully cut into small pieces (4-9 mm^2) and washed with 70% ethanol. The tissues were immersed in X-Gluc substrate solution containing 50 mM sodium phosphate buffer (pH 7.0), 0.5 mg/ml 5-bromo-4-chloro-3–indolylglucuronide (X-Gluc), and 0.05% Triton X-100, and then incubated at 37ºC for 1 h (or overnight). After incubation, the stained tissues were washed several times with 70% ethanol to remove residual chlorophyll, and visualized using an Olympus SZ 4045TR stereomicroscope

Results and discussion

The ATP fragment is able to confer a high level of gene expression in trichomes

We have previously observed that the *ATCYS-3A* gene (At4g14880) from *A. thaliana* was preferentially highly expressed in leaf and stem trichomes (Gotor *et al.*, 1997; Gutiérrez- Alcalá *et al.*, 2000). Based on this observation, we have isolated the DNA regulatory fragment of this gene. The *ATCYS-3A* gene has been renamed *OASA1* by others, and therefore, we have selected this nomenclature in the present article (Jost *et al.*, 2000). The DNA regulatory fragment is 1810 nucleotides in length and is named as ATP fragment. The isolated fragment consists of 1435 bp of the genomic region upstream of *OASA1*, and 375 bp of the 5´UTR containing the first exon, the first intron, and 22 bp of the second exon of the gene (Fig. 1A). The transcription initiation site was determined by primer extension being the first nucleotide

numbered +1 (Fig. 1B), and the intron/exon boundaries were determined by comparison with the cDNA sequence (Barroso *et al*, 1995). The ATP fragment was fused to the green fluorescent protein (GFP) and β-glucuronidase (GUS) reporter genes, and the constructs for plant transformation were named pATP-GFP and pATP-GUS, respectively. Several transgenic *A. thaliana* plants were obtained and homozygous lines analyzed by laser confocal microscopy for *in vivo* GFP detection or by light microscopy for histochemical GUS detection. Intact leaves from two-week old *A. thaliana* plants were analyzed and strong GFP fluorescent emissions (pseudocolored green) were largely detected in the trichomes but not in control plants (Fig. 2A-B). We observed GFP inside the nuclei of trichomes, in a thin layer of cytoplasm underneath the cell wall, and in cytoplasmic strands across the vacuoles. Weaker signals were also detected in a thin layer of cytoplasm in the epidermal cells, although at a lesser intensity than in trichomes (Fig. 2B-C). This observation is in agreement with studies of *in situ* localization of the *OASA1* mRNA in leaves, showing a low constitutive expression in epidermal and mesophyll cells (Gotor *et al.*, 1997). Wild-type plants did not show any fluorescence under GFP band detection (493-540 nm) and only showed auto-fluorescence in the cell walls of the epidermal and trichome cells and in the chloroplasts of mesophyll cells (pseudocolored red) (Fig. 2A). The same results were obtained by histochemical staining of the ß-glucuronidase activity in the pATP-GUS transformed lines (Fig.2D-E). High GUS activity was observed in the basal and central regions of the leaf trichomes and a very low activity was detected in a thin layer of cytoplasm in the epidermal cells.

Therefore, the ATP fragment is largely expressed but is not trichome-specific in *A. thaliana.*

ATP fragment deletion analysis

Database searches for regulatory elements in the ATP fragment by PLACE (http:://www.dna.affrc.go.jp/htdocs/PLACE/) revealed a putative TATA box at the –48 position, several MYB recognition sites, and other putative regulatory binding sites. MYB factors are transcriptional regulators involved in developmental control, cell fate, and identity, such as *GLABROUS1*, which control trichome formation (Oppenheimer *et al.*, 1991). These MYB recognition factors have been detected in the promoters of trichome-expressed genes, such as in cotton fibers *LTP3* and *RDL1* and tobacco *CYP71D16* (Liu et at, 2000; Wang *et al.*, 2004b; Wang *et al.*, 2002). Although there are no studies about *cis*-responsive elements in the *LTP3* and *CYP71D16* promoters involved in trichome expression, the essential role of the L1 box and MYB binding motif in trichome expression, localized to the *RDL1* promoter region, was previously described (Wang *et al.*, 2004b).

To identify the regulatory regions involved in trichome expression in the ATP fragment, we have performed successive 5´ and 3´ deletions of this fragment to generate seven fusion constructs of GFP (Fig. 3A). In the ATP2 deletion, a largely 5´-upstream fragment containing the adjacent genes of *OASA1* has been removed. This deletion contains 419 bp of the intergenic region between the *OASA1* and *ferredoxin* genes and 375 bp of the 5´-UTR. The ATP2I, ATP2II, and ATP6 constructs are

additional deletions at the 5´-end. Fluorescence analyses in several transgenic lines of each deletion construct showed that ATP and ATP2 drive the same expression patterns in the *A. thaliana* trichome. ATP2I and ATP2II deletion constructs also showed similar GFP fluorescence intensities compared to the full-length promoter in trichomes, while for ATP6 lines, no detectable fluorescence was observed (Fig. 3). Therefore, the DNA region from –269 to –66 contains the regulatory elements required for trichome expression (Fig.3Be). Deletion analysis at the 3´-end of the ATP fragment helped identify a domain from +112 to +375 that is also essential for trichome expression and is comprised mainly of the first intron localized in the 5´-UTR (Fig. 3Bf). This localization of regulatory elements in the ATP fragment is similar to that obtained in the promoter region of the *RDL1* gene. Database searches for regulatory elements in these ATP regions by PLACE showed that the region between –268 to –66 contains four MYB motifs, two of which are separated by 16 nucleotides. However, this analysis does not predict any L1 box motif within the *RDL1* promoter to be essential for trichome expression (Wang *et al.*, 2004b).

The presence of introns at the 5´-UTR region is a common feature of several *A. thaliana* trichome-expressed genes, such as the MADS-box *AGL16* gene (Alvarez-Builla *et al.*, 2000), the ionizing radiation induced *GR1* gene (Deveux *et al.*, 2000), the metallothionein *MT2a* gene (García-Hernández *et al.*, 1998), and the wound-responsive *WR3* gene (León *et al.*, 1998). This 5´-UTR region can play an important role in the regulation of gene expression by regulating mRNA stability, the efficiency of translation, or can directly impact transcriptional regulation (Bailey-Serres and Gallie, 1998). It has been demonstrated that enhancer elements present in the introns

of the 5´-UTR are involved in transcriptional regulation (Gidekel *et al.*, 1996; Maas *et al.*, 1991; Plesse *et al.*, 2001; Morello *et al.*, 2002). However, introns may influence and enhance eukaryotic gene expression by multiple mechanisms (Le Hir *et al.*, 2003). The transcription factors GL1 and GaMYB2 contain a MYB motif that is present in the first intron of these genes that acts as an enhancer element in trichome cells (Wang *et al.*, 2004b). The intron region of the *OASA1* gene present in the ATP fragment appears to also be essential for trichome expression since the ATP3 construct did not show GFP fluorescence (Fig. 3Bf). PLACE searches indicated that this intronic region between +112 to +375 contains three putative MYB motifs, although the position of these motifs was not conserved when compared with the intronic elements of *GL1* and *GaMYB2* that are localized at the same position (i.e., 22-23 bp upstream of the second exon) (Wang *et al.*, 2004b).

The Arabidopsis ATP fragment is functional in glandular trichomes

A. thaliana trichomes are unicellular, non-secretory structures. Despite their morphological differences with the glandular trichomes, we wanted to test whether the ATP fragment could drive high-level gene expression in these structures. *M.* x *piperita* leaf disks were transformed with the pATP-GFP and pATP-GUS constructs (via *A. tumefaciens*) and several kanamycin-resistant peppermint plants were generated. Peppermint leaves contain two types of secretory trichomes, peltate and capitate glands. Peltate trichomes are composed of a basal cell, a short stalk cell, and a broad head that consists of eight secretory cells arranged in a single layer

(Ameluxen, 1965). The capitate trichomes consist of a basal cell, one stalk cell, and a head composed of one or two cells (Fahn, 2000). GFP analysis of the transformed plants showed high levels of expression in the head and stalk cells of the capitate trichomes and in the head of the peltates. Other epidermal and mesophyll cells did not show detectable GFP fluorescence (Fig. 4A-H). Analysis of the pATP-GUS-transformed peppermint lines confirmed the same pattern of expression in capitate and peltate trichomes (Fig. 4I-K) with sometime, a little diffusion of the blue color in the surrounding epidermis.

N. tabacum plants containing glandular trichomes were also transformed with the two constructs. Such glandular trichomes comprise a very long-stalk and pluricellular head. Due to the strong auto-fluorescence of these trichomes, *in vivo* analysis of the GFP reporter could not be performed. However, analysis of GUS activity in the transformed lines showed significant expression in all trichome cells with a very strong signal in the glandular head (Fig. 4L-M).

In conclusion, the isolated ATP fragment is able to confer high levels of gene expression in different types of plant trichomes. Biotechnological applications of this promoter on disease resistance have been attempted by expression of glucanase enzymes from *Trichoderma harzianum*, showing antifungal activities, under control of the ATP fragment. Stable high-level expression of these defense-related genes preferentially in trichome shows increased resistance to necrotrophic fungi (Calo *et al*, unpublished). Its additional functionality in glandular trichomes suggests potential biotechnological applications for this regulatory DNA fragment. In peppermint, essential oils are stocked between the cell wall and the detached cuticle with a very

low level of evaporation (Gershenzon *et al.,* 2000). At the opposite, in tobacco, essential oils and polysaccharides are directly pour out the head-cells, giving a resin-like liquid on the leaves. Such differences in the chemical secretion pathway could permit a very fine-tuning of the biotechnological strategies.

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Figure Legends

Fig. 1. Schematic representation of the ATP fragment and mapping of the transcription start site. A) Diagram of the 1810-nucleotide ATP fragment, including the 5´-end of the *OASA1* gene and upstream genes. Blue depicts the intergenic regions, red depicts the 5´- and 3´-untranslated regions, and the striped box corresponds to the intronic region of *OASA1*. B) The transcription start site was determined by primer extension analysis. The amplified band after reverse transcription co-migrates with a guanidine residue and is marked by an asterisk in the bottom sequence.

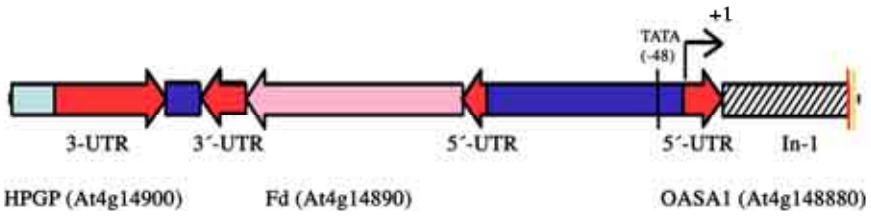
Fig. 2. Expression analysis of the ATP fragment in *Arabidopsis* trichomes. Imaging of the GFP and GUS reporter genes fused to the ATP fragment. A) GFP imaging of a wild-type *Arabidopsis* leaf in a single optical section. B) Maximum projection of 20 optical sections of an *Arabidopsis* leaf from pATP-GFP transgenic lines. C) GFP imaging of an *Arabidopsis* leaf trichome from pATP-GFP transgenic lines in a single optical section. D) Histochemical GUS staining of a wild-type *Arabidopsis* leaf. E) GUS localization in a transgenic pATP-GUS *Arabidopsis* leaf.

Fig. 3. Identification of regulatory elements in the ATP fragment. A) Schematic diagram of the 5´- and 3´-deletion series of the ATP fragment. The GFP reporter gene (green) was fused to deleted ATP fragments as indicated in parenthesis. The arrow shows the initiation start site. B) GFP fluorescence in deletion lines. GFP reporters were imaged in single

optical sections in leaf trichomes from the following transgenic lines: a) ATP, b) ATP2, c) ATP2I, d) ATP2II, e) ATP6, f) ATP3, g) ATP4, h) ATP5.

Fig. 4. Expression analysis of the ATP fragment in glandular trichomes. Fluorescence and transmitted light imaging of GFP and GUS expression in trichomes. A-B) Peltate trichome of pATP-GFP transgenic *M.* x *piperita*. C-D) Capitate trichomes of the same plants. E-F) Peltate trichomes of control plants. G-H) Capitate trichomes of control plants. I-K) Peltate and capitate trichomes of pATP-GUS transgenic *M.* x *piperita*. L) Secretory trichomes of control *N. tabacum*. M) Secretory trichomes of pATP-GUS transgenic *N. tabacum*.

A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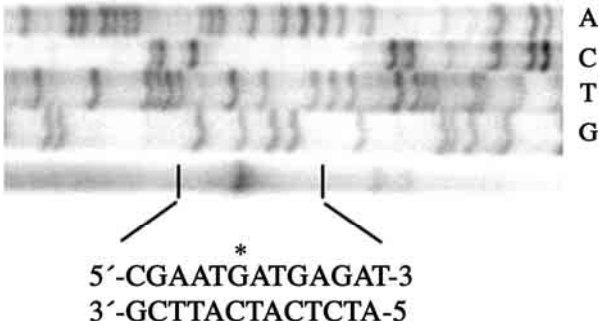
TATA
(-48)
3-UTR
3´-UTR
5´-UTR
5´-UTR
In-1
HPGP (At4g14900)
Fd (At4g14890)
OASA1 (At4g148880)

B


A
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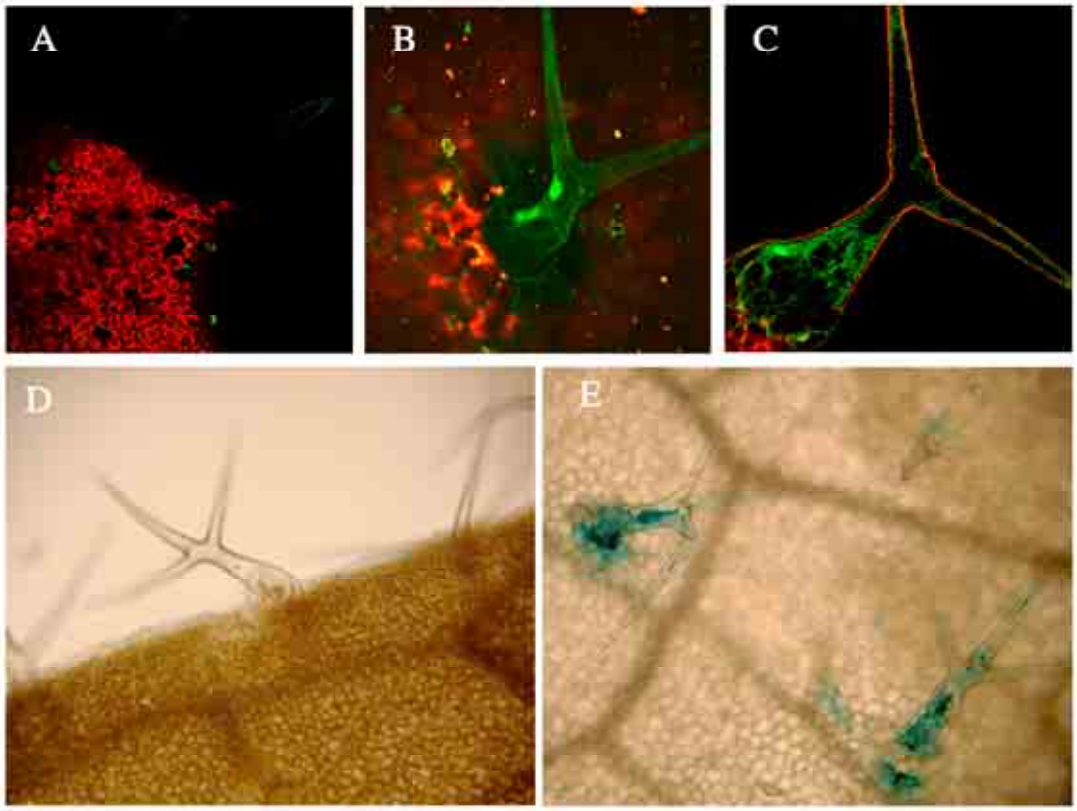
3´-GCTTACTACTCTA-5


A
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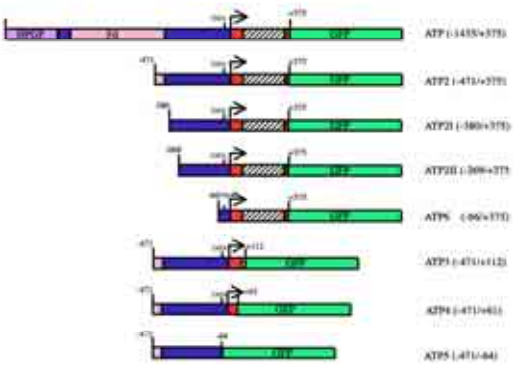
A



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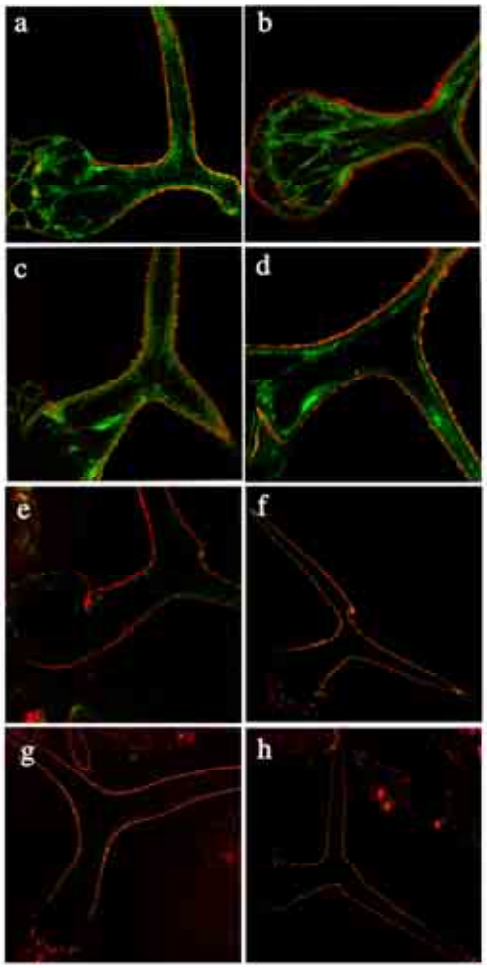

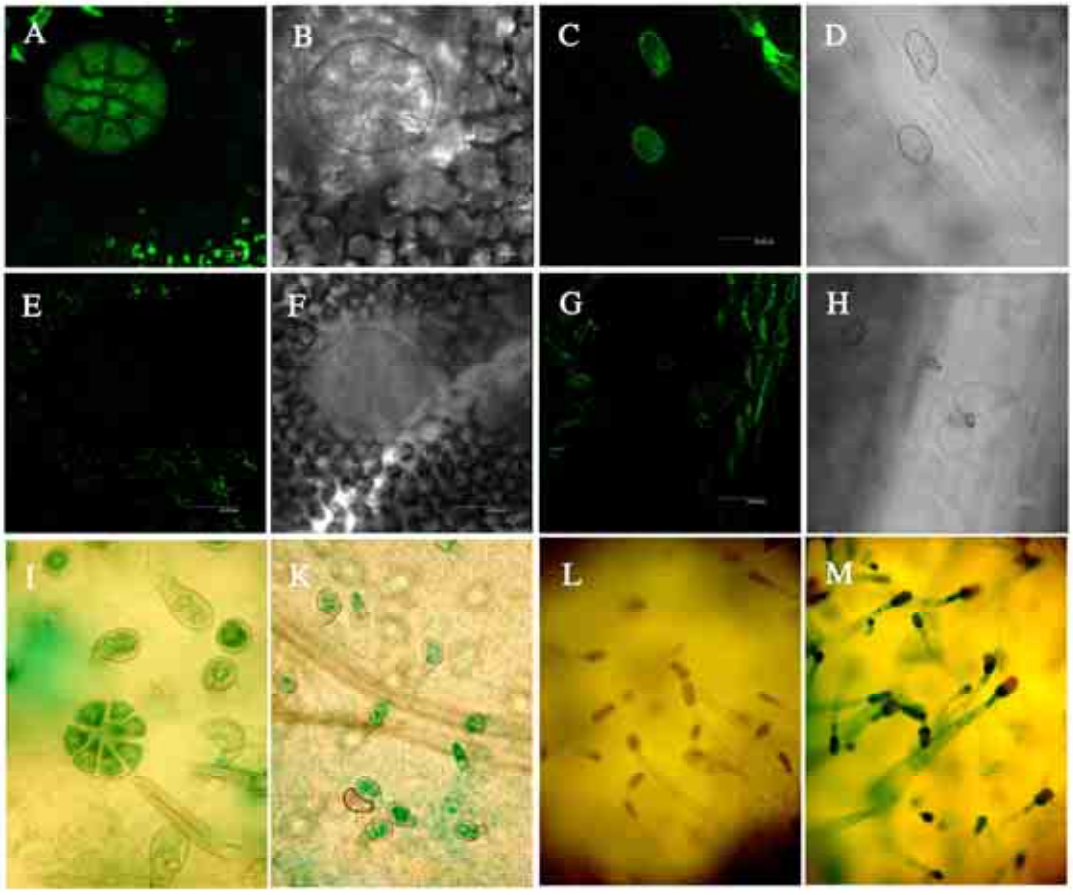
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